Research paper

EHD1 impairs decidualization by regulating the Wnt4/β-catenin signaling pathway in recurrent implantation failure

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ABSTRACT

Background: Recurrent implantation failure (RIF) remains a critical and challenging problem in assisted reproductive technology mainly due to impaired decidualization. The endocytic and transcytotic activity in the endometrium are crucial for decidualization. The most representative endocytic gene is the C-terminal Eps15 homology domain-containing 1 (EHD1), but whether EHD1-mediated endocytic function is responsible for embryo implantation during decidualization remains unclear.

Methods: A transcriptomic analysis was performed to evaluate the differentially expressed genes between the fertile control and RIF group. The expression and location of EHD1 in endometrial tissues were further examined by IHC, qRT-PCR and Western blotting. The transduction of an EHD1 recombinant adenovirus into human endometrial stromal cells was performed to investigate relevant decidualization marker genes. Additionally, a microarray analysis following the adenovirus-mediated overexpression of EHD1 was conducted to identify EHD1-related changes in HESCs, and the potential molecular mechanisms were further confirmed through immunofluorescence and coimmunoprecipitation analyses.

Findings: An RNA-seq analysis demonstrated that EHD1 expression was significantly higher in the mid-secretory endometrium of the RIF group than in that of the fertile control group. The analysis of the menstrual cycle showed that expression of EHD1 increased in the mid-proliferative phase and showed a gradual decrease in the mid-secretory and decidual phases. Furthermore, EHD1 overexpression impaired decidualization by suppressing the expression of prolactin and insulin-like growth factor binding protein-1 and the formation of the cytoskeleton. The mechanistic analysis revealed the EHD1 regulated LRP5/6 protein function through the endocytic pathway, and subsequently suppressed the Wnt4/β-catenin pathway during decidualization. In addition, a Wnt4 agonist improved an impaired decidualization process.

Interpretation: Regulation of the EHD1-Wnt4 pathway might serve as a promising therapeutic strategy for improving endometrial receptivity in RIF women.

Research in context

Evidence before the study

Recurrent implantation failure (RIF) remains a critical and challenging problem in assisted reproductive technology mainly because of impaired decidualization. Endocytic and transcytotic activity in the endometrium are crucial for impaired decidualization. The most representative endocytic genes is the C-terminal Eps15 homology domain-containing 1 (EHD1). Whether the EHD1-

mediated endocytic function is responsible for embryo implantation during decidualization remains to be determined.

Added value of this study

Our study demonstrated that EHD1 is a critical repressor of the steroid hormone induction of decidualization in RIF patients. EHD1 interacts with Wnt4, leading to the suppression of Wnt4/β-catenin signaling and impairing decidualization in the endometrium.

Implication of all the available evidence

Our study provides new insight into the molecular mechanism of EHD1, showing that it might act as a regulator that controls cell membrane transport that is critical for decidualization. From a therapeutic perspective, therapies targeting EHD1 could be applicable for the treatment of implantation failure caused by impaired decidualization.

1. Introduction

Recurrent implantation failure (RIF) is generally defined as failure to achieve clinical pregnancy after the transfer of at least four good quality embryos during three consecutive fresh or frozen in vitro fertilization (IVF) cycles [1]. Endometrial dysfunction and/or poor embryo quality are considered the most crucial causes of RIF [2,3]. However, most of these women cannot become pregnant even when they undergo transfers with high-quality embryos [4], and increased lines of evidence indicate that inadequate and impaired decidualization is the primary cause of RIF [4,5]. Decidualization is the differentiation of elongated, fibroblast-like mesenchymal cells into rounded, epithelioid-like cells under stimulation by progestogen and cyclic adenosine monophosphate (c-AMP) [6,7]. The morphological differentiation and hypermetabolic state of human endometrial stromal cells (HESCs) make them ready for subsequent embryo implantation [8–10]. However, few studies have investigated the molecular mechanisms underlying impaired decidualization during RIF.

We performed an mRNA sequence analysis to investigate the potential mechanisms of impaired decidualization in the endometrial tissues of RIF patients and found that a series of genes related to endocytic function were obviously changed. Among the 2140 differentially expressed genes (DEGs), the most representative endocytic gene was C-terminal Eps15 homology domain-containing 1 (EHD1). The EHD1 molecule acts in early endocytic membrane fusion and regulates the membrane trafficking of recycling endosomes [11,12]. EHD1, as a regulator of endocytosis, plays an important role in cell proliferation, differentiation and apoptosis among other cellular activities [13–15]. Importantly, EHD proteins play a vital role in regulating the transport of receptors, such as epithelial growth factor receptor [16], insulin-like growth factor receptor [17] and colony-stimulating factor-1 receptor [18]. Quinn et al. [19] reported that endocytic and transcytotic activity in the endometrium are crucial for embryo implantation, but whether EHD1-mediated endocytic function is responsible for embryo implantation during decidualization remains unclear.

We hypothesized that EHD1 is involved in the pathogenesis of RIF, because aberrantly increased expression of EHD1 affects decidualization by regulating the endocytosis of its downstream cell membrane receptor. We demonstrated that increased EHD1 expression decreases the secretion of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP-1) and inhibits cytoskeletal formation during decidualization. We further showed that EHD1 regulates low-density-lipoprotein receptor-related proteins 5 and 6 (LRP5/6) coreceptor via the endocytic pathway and thereby represses decidualization by downregulating Wingless-related MMTV integration site 4 (Wnt4) pathway activity. Additionally, the Wnt4 agonist alleviates the impaired decidualization of HESCs.

2. Materials and methods

2.1. Ethics approval

This study was carried out according to the Recommendations of Guidelines for Clinical Trials by the Ethics Committee of the Drum Tower Hospital. Informed consent was obtained from all participants before any study-related procedure was performed. This study was carried out according to the approval of Construction and Management of the Nanjing Multic-center Biobank Project (No. 2013-081-01, Registered Dec10,2013)

2.2. Patients

From July 2017 to December 2018, 54 women (aged between 23 and 39 years) who were treated at the Reproductive Medicine Center of Affiliated Drum Tower Hospital were enrolled in this study. All women exhibited a regular menstrual cycle ranging from 28–33 days, and none of them received hormonal therapy in the last three months. The exclusion criteria included polycystic ovarian syndrome (PCOS), hydrosalpinx, endometriosis, adenomyosis, endometrial hyperplasia or endometrial polyps. The details of these patients are summarized in Table 1. Twenty-seven RIF women were enrolled in the present study. The control group included 27 women whose infertility was due to male factors, and these women were confirmed to have achieved a clinical pregnancy after their first or second IVF treatments. All women were monitored for ovulation by transvaginal ultrasound during the menstrual cycle, and the endometrium in the mid-secretory phase were obtained via pipe suction curettage in 5–7 days after ovulation. At the same time, endometrial samples from 10 women were obtained from the mid-secretory phase for isolation of primary HESCs.

2.3. RNA-seq and data analysis

Total RNA was isolated and used for RNA-seq analysis, and the cDNA library was constructed by Beijing Genomics Institute using the Illumina HiSeq X platform (Shenzhen, China). High-quality reads were aligned to the human reference genome (GRCh38) using Bowtie2. The expression levels of each genes were normalized to fragments per kilobase of exon model (FPKM) per million mapped reads using RNA-Seq by Expectation Maximization (RSEM). The principal components analysis (PCA) was performed using the princomp function and the graphics were drawn using the ggplot2 package in R software. The hierarchical cluster analysis using the pheatmap function in R software were used to assess the gene expression (FPKM values) among the samples. The DEGs related to endocytosis or the Wnt pathway were further analyzed using the Dr-TOM online analysis tool with the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

2.4. Cell culture, steroid hormones, and agonist

HESCs were isolated from the mid-secretory phase, and processed according to Sun et al. [20] with minor modifications. First, the endometrial tissues were minced and enzymatically digested with 0.15% (w/v) collagenase I (Worthington Biochemical Corp, Lakewood, NJ, USA) for 30 min at 37 °C. Next, the digested tissues were filtered through a 30 μm-sieve gauze to separate the stromal cells from the glands. The endometrial stromal cells were maintained in DMEM/F12 (HyClone, Thermo Scientific, South Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo Scientific, South Logan, UT, USA), 100

Table 1
Clinical characteristics of women enrolled in the present study.

Variables	Normal groups (n = 27)	RIF patients (n = 27)	Mid-secretory phase endometrium for isolation of primary HESCs (n = 10)	p value
Age (y),	30.7 ± 3.0	30.4 ± 3.7	29.8 ± 2.7	0.752
BMI (kg/m²)	22.2 ± 3.6	23.7 ± 34	21.8 ± 3.6	0.122 [a]
FSH (mIU/ml)	7.8 ± 1.8	7.1 ± 2.2	8.2 ± 2.3	0.210 [a]
LH (mIU/ml)	4.7 ± 2.0	4.6 ± 3.2	4.2 ± 1.1	0.909 [a]
Estradiol (pg/ml)	36.8 ± 16.9	36.1 ± 19.4	35.8 ± 21.7	0.777 [a]
Number of embryo transfer, n%	3 (12/27, 44.4%) 4 (6/27, 22.2%) 5 (7/27, 26.0%) 6 (2/27, 7.4%)	1 (21/27, 77.8%) 2 (6/27, 22.2%)		<0.001 [b]
Number of embryo per transfer, n%	1.8 ± 0.6	1.6 ± 0.5		0.189 [c]
Average score of transferred embryos	7.7 ± 0.8	7.8 ± 0.9		0.685 [c]

These continuous data were expressed as mean ± SD and a normal distribution test was performed for normality before using the student's t-test. The categorical variables were expressed by n%. p<0.05 were considered with significant statistical difference.

IU/mL penicillin and $100\,\mu g$/mL streptomycin, seeded into culture dishes and incubated at 37 °C in 5% CO2. After 48 h, decidualization was induced in HESCs as described by Brosen et al. [21]. HESCs were cultured in phenol red-free DMEM/F12 (Gibco BRL/Invitrogen, Carlsbad, CA, USA) containing 2.5% charcoal/dextran-treated FBS (Thermo Scientific, South Logan, UT, USA), 100 IU/ml penicillin, and $100\,\mu g$/ml streptomycin. Treatment included 0.5 mM 8-Br-cAMP (B7880, Sigma-Aldrich, St. Louis, Mo, USA), $1\,\mu M$ methoxyprogesterone acetate (MPA) (M1629, Sigma-Aldrich, St. Louis, Mo, USA) and/or $10\,\mu M$ Wnt4 agonist (AG-L-67051, sc-222416, Santa Cruz Biotechnology, Dallas, TX, USA) .

2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from HESCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Purified total RNA $(1\,\mu g)$ was reverse-transcribed into cDNA using random primers with a Prime Script RT reagent kit (Takara). qRT-PCR was performed using a SYBR green PCR kit and a MyiQ Single Color Real-time PCR Detection System ((BIO-RAD, Hercules, CA, USA). The following primers were used for the indicated genes: EHD1, 5′-ATGTTCAGCTGGGTCAGCAA-3′ and 5′-TTGTCGAAGTCAGCGTCCTC-3′; EHD2, 5′-TGGTGCGAGTTCACGCTTAC-3′ and 5′ -TGACGGGCAGTTTGAGGAT-3′; EHD3, 5′- GCT-CATGGTGCTAGTGCG −3′ and 5′- CGGTGACAGGGTGTAGAA-GAT −3′;EHD4, 5′- GCAAGACCACCTTCATCAGA −3′ and 5′-GTCCACGACTAAAGCATTCC-3′; PRL, 5′-CACTACATCCATAACCTCTC-3′ and 5′-ATGCTGACTATCAAGCTCAG-3′; IGFBP1, 5′-TATGATGGCTCGAAGGCTCTC-3′ and 5′-GTAGACGCACCAGCAGCAG-3′; and 18SrRNA, 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-CTGGAATTACCGCGGCT-3′. The PCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Reactions were run in duplicate using RNA samples from three independent experiments. The fold change in expression of each gene was calculated using the $2^{-\Delta Ct}$ (ΔCt, relative cycle threshold compared with 18S) method, with 18S rRNA as an internal control.

2.6. Construction of plasmid and adenovirus vectors

The pCS2-myc-Wnt4 expression plasmid was constructed using the primers 5′-GCCCGAATTCCCAGTCCCCGCTCGTGCCTGCGTTCGCTG-3′ and 5′-CCGCGGTACCTCATCGGCACGTGTGCAACTCCACG-3′ with a Gibson assembly cloning kit (NEB, USA). Adenovirus vectors harboring the full-length EHD1 gene (Ad-Flag-EHD1, NCBI Reference Sequence: NM_001282444.2) were generated using RAPAd® CMV Adenoviral Expression Systems (Cell Biolabs lnc, USA) according to the manufacturer's recommendation. The adenovirus bearing LacZ vector (Ad-LacZ) was obtained from Clontech (Palo Alto, CA, USA) and was used as a control in the adenovirus-mediated EHD1 overexpression experiments. The virus was packaged and amplified in HEK293A cells and purified using CsCl banding.

2.7. Coimmunoprecipitation (co-IP)

HEK293T cells were transiently cotransfected with the indicated plasmids. After transfection for 48 h, the cells were lysed in whole-cell lysis buffer (50 mM Tris-HCl pH=7.6, 150 mM NaCl, and 1.0% NP-40) containing a protease inhibitor cocktail (Sigma-Aldrich). Next, $500\,\mu g$ of each cell lysates was incubated with protein A/G PLUS-agarose beads (Abmart, Shanghai, China) at 4 °C for 2 h. Then, the lysates were incubated with $30\,\mu l$ of anti-Flag M2 beads (Sigma-Aldrich) at 4 °C overnight with constant shaking. The samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene difluoride membranes

(Millipore, Billerica, USA), and analyzed by Western blot using an Flag-HRP antibody (1:10000; RRID:AB_439702) and an anti-Myc-HRP antibody (1:5000; Thermo Scientific, RRID:AB_779857).

2.8. Western blot analysis

Proteins from the tissues or cells were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were then incubated overnight at 4 °C with antibodies against the proteins of interest, including EHD1 (1:2000; ab109311; Abcam; RRID:AB_10859459), EHD2 (1:2000; ab23935; Abcam; RRID:AB_2097328), EHD3 (1:1000; 25320-1-AP; Proteintech), EHD4 (1:1000; 11382-2-AP; Proteintech),Wnt4 (1:1000; sc-376279; Santa Cruz; RRID:AB_10986273), β-catenin (1:1000; ab32572; Abcam; AB_725966) and GAPDH (1:10000; AP0063; Bioworld; RRID:AB_2651132) overnight at 4 °C. Detection was performed using an enhanced chemiluminescence kit (Millipore, Billerica, USA), and the expression of each protein was normalized to the expression of GAPDH in the corresponding sample.

2.9. Immunofluorescence staining

HESCs grown in 24-well cell culture plates, and endometrial tissue were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (w/v) for 20 min at room temperature. After three 5-min washes with PBS, the fixed coverslips were permeabilized in PBS with 0.1% Triton X-100 for 5 min at room temperature. Nonspecific sites were blocked in PBS for 1 h at 37 °C. The cells were incubated with fluorescein isothiocyanate-labeled phalloidin (1:200, P5282, Sigma-Aldrich) and an anti- flag antibody (1:200; 8146; Cell Signaling Technology; RRID: AB_10950495) at 4 °C overnight, the endocytic function was determined using anti-Rab11 (1:200; 5589S; Cell Signaling Technology; RRID: AB_10693925), anti-LRP5(1:100; sc-390267; Santa Cruz),and anti-LRP6 (1:100; sc-25317; Santa Cruz; RRID: AB_627894). After washing with BSA/PBS (three times), the coverslips were further incubated with Alexa Fluor 594-conjugated donkey anti-goat IgG (1:200, Invitrogen; RRID: AB_141359) and Alexa Fluor 488-conjugated goat antirabbit IgG (1:200, Invitrogen; RRID: AB_141607). Nuclei were stained with 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich). Finally, images were captured by fluorescence confocal microscopy (Olympus, FV10i).

2.10. Immunohistochemistry

Human endometrial tissue sections (5 μm) on slides were deparaffinized in xylene and ethanol. Endogenous peroxidases were blocked by means of incubation with 3% H2O2 for 10 min. Slides were blocked with 1.5% normal goat or rabbit blocking serum for 45 min at room temperature and the sections were incubated overnight at 4 °C with EHD1 (1:2000; ab109311; Abcam; RRID:AB_10859459) and Wnt4(1:1000; sc-376279; Santa Cruz; RRID:AB_10986273). After being washed with PBS, the sections were incubated with a goat anti-rabbit secondary antibody at 37 °C for 30 min. Finally, the sections were stained with 3, 3′-diaminobenzidine and counterstained with hematoxylin. Nonspecific rabbit IgG and goat IgG were used as negative controls, and the staining of these control samples was performed alongside that of the experimental sections. Nonspecific staining was not detected in the controls.

2.11. Detection of prolactin levels in cultured supernatants

PRL levels were measured using a Mini-Vidas V.B02.96 system with a Vidas Prolactin kit (bioMerieux, France). The lower limit of detection using the kit was 0.25 ng/ml.

2.12. Statistical analysis

Statistical analyses were performed using Prism version 7 software (GraphPad, LaJolla, CA). Continuous variables are shown as the mean ±SD, and differences between groups were tested by the Student's *t*-test or one-way analysis of variance. All *p*-values < 0.05 were considered significant.

3. Results

3.1. Demographics

As shown in Table 1, no significant differences in age, BMI, basal FSH, LH or Estradiol levels, the number of embryos per transfer and the average score of the transferred embryos were found between the control and RIF groups. The number of transferred embryos in the RIF group was significantly ($p < 0.001$) higher than that in the control group.

3.2. RNA-Seq data analysis

The PCA of all the normalized data showed two significant clusters in the control (blue dots) and RIF (yellow dots) groups, and indicated that the similarity of the samples belonging to the same group was higher than that found among samples belonging to different groups (Fig. 1a). A total of 18,631 DEGs were detected between the two groups; in addition, 699 genes were only expressed in the control group, and 633 genes were only expressed in the RIF group (Fig. 1b). Among the probe sets, 2140 DEGs in the RIF group exhibited a fold change greater than 2 and an adjusted p-value less than 0.05. Among the 2140 DEGs, 966 were upregulated, and 1174 were downregulated (Fig. 1c). The DEGs related to endocytic function were clustered using a hierarchical clustering algorithm (Fig. 1d), and KEGG pathway and Go analyses clustered these DEGs into groups based on their biological processes, molecular functions and cellular components (Fig. 1e, 1f). 'Endocytosis' and 'regulation of receptor-mediated endocytosis' comprised the majority of the enriched GO terms. In particular, the level of EHD1, a representative protein that regulates endocytic function, was significantly increased in the RIF group compared with the control group.

3.3. The expression of EHD1 is aberrantly increased in RIF patients

The above-described findings encouraged us to further investigate the role of EHD1 in the endometrium. Immunohistochemistry analysis showed that EHD1 was expressed in both stromal and epithelial tissues, and significantly higher expression of EHD1 was detected in the RIF group with the control group. High expression of EHD1 was observed in epithelial cells in both the control and RIF groups, but the difference between the two groups was not statistically significant. Interestingly, the level of EHD1 expression in stroma cells was significantly increased in the RIF group compared with the control group (Fig. 2a) (Supplementary Figure 1a). Furthermore, the Western blot shown in Fig. 2b also suggested that the RIF presented an increased endometrial EHD1 level (by greater than 2-fold) compared with the control group. We thus investigated the aberrant expression of EHD1 within stromal cells in the RIF group. The decidualization of human endometrial stromal cells is a crucial step for making a uterus receptive to an embryo and these findings support the potential role of EHD1 during decidualization in RIF.
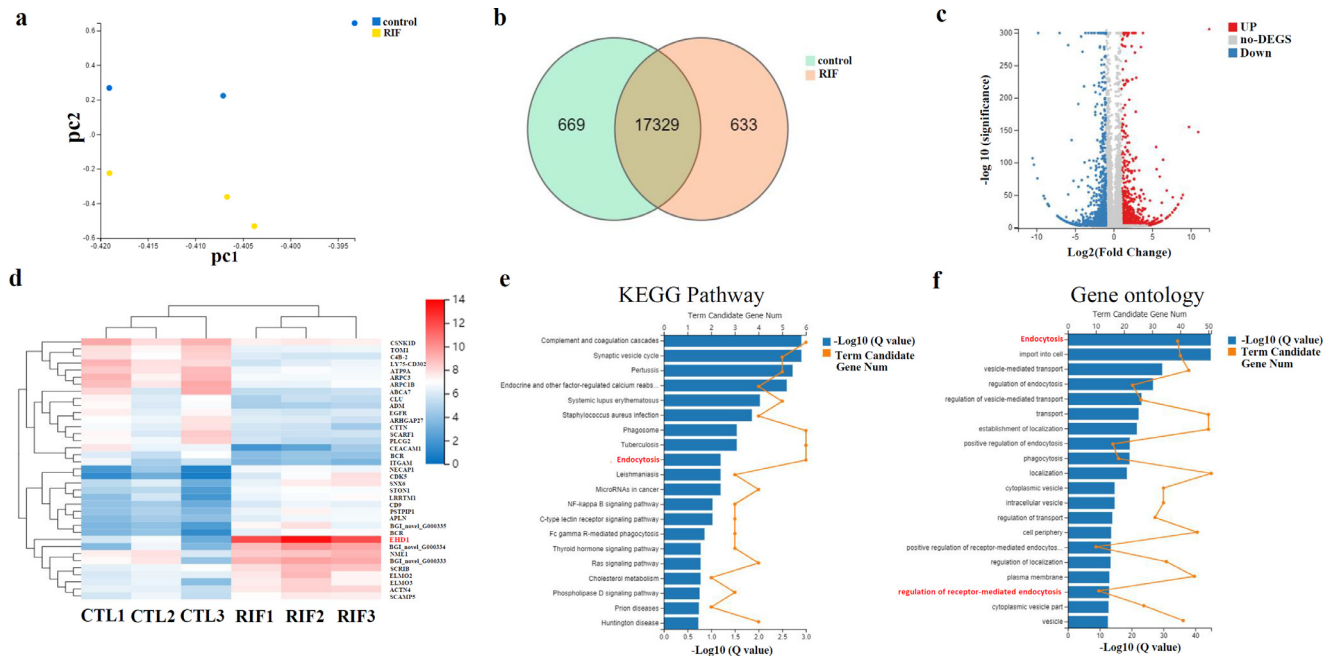
Fig. 1. RNA-seq results between the recurrent implantation failure (RIF) and control groups. (a) 2D scatter plot of the principal component analysis (PCA) results. Samples in the control group ($n = 3$) and RIF group ($n = 3$) are represented as blue dots and yellow dots, respectively. (b) Venn diagrams showing the overlapping genes and differentially expressed genes (DEGs). (c) Volcano plot of DEGs. Up- and downregulated DEGs with $p < 0.001$ and a fold change >2 are highlighted in light blue and light red, respectively. (d) Hierarchical clustering of DEGs between the control group and the RIF group. The sample parts are listed on the primary x-axis, while DEGs are listed on the y-axis with their expression ratios (log2) expressed per the color gradient shown. (e) KEGG pathway analysis showed the top ranking pathway associated with endocytosis. (f) Gene Ontology (GO) functional classification of DEGs. The x-axis represents the number of DEGs (the number is presented as its square root value), and the y-axis represents GO terms. GO terms related to endocytosis are highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
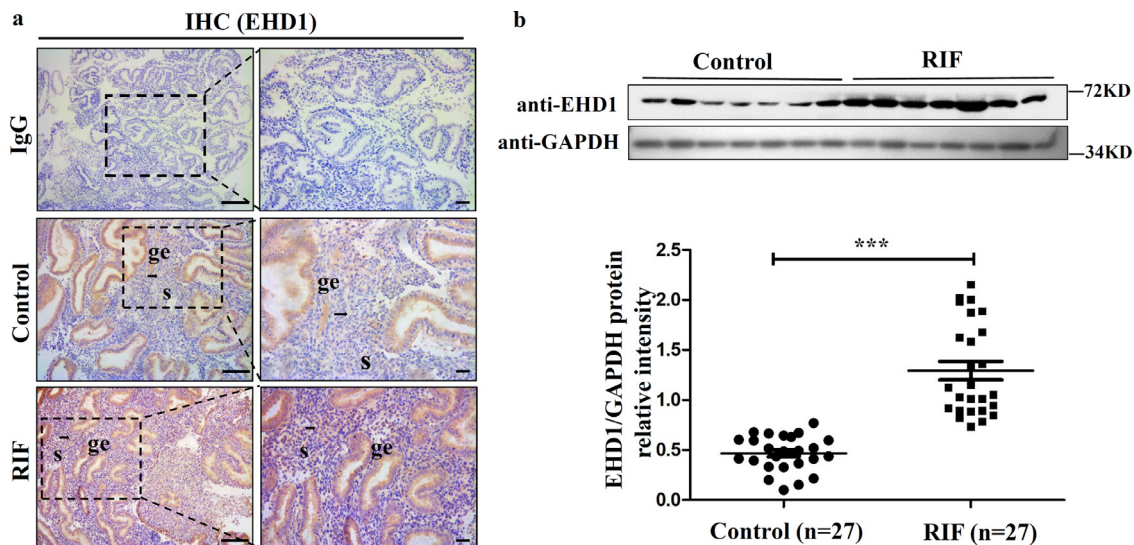

Fig. 2. Aberrant expression of EHD1 in the endometria of RIF patients. (a) Immunohistochemical analysis was performed using an EHD1 antibody. Endometrial tissue samples from fertile women and RIF patients are shown at $100\times$ (left panel) and $200\times$ (right panel) magnification. Scale bars: $200\,\mu$m. s, stroma; ge, glandular epithelium. Arrows show the increased EHD1 conjugates in the stroma. (b) The total EHD1 protein levels were normalized to total GAPDH levels in the Western blot analysis ($n = 27$, ***$p < 0.001$, student's t-test).

3.4. EHD1 expression is inhibited under stimulation by 8-Br-cAMP and MPA

First, to investigate the role of the EHD family of proteins in the regulation of decidualization, we examined the mRNA and protein levels of EHD1, EHD2, EHD3, and EHD4 during the menstrual cycle. The EHD1 mRNA and protein expression levels showed a gradual decrease over the menstrual cycle, but no significant changes in the EHD2, EHD3 or EHD4 mRNA and protein levels were observed (Supplementary Fig. 2a and b). An immunolocalization analysis suggested that the EHD1 protein abundance in HESCs was lowest at the decidual phase during the menstrual cycle (Fig. 3a and b). Furthermore, we examined EHD1 expression in HESCs in response to stimulation with both 8-Br-cAMP and MPA, which are well known to induce decidualization in HESCs in vitro. The results showed that 8-Br-cAMP and MPA gradually inhibited EHD1
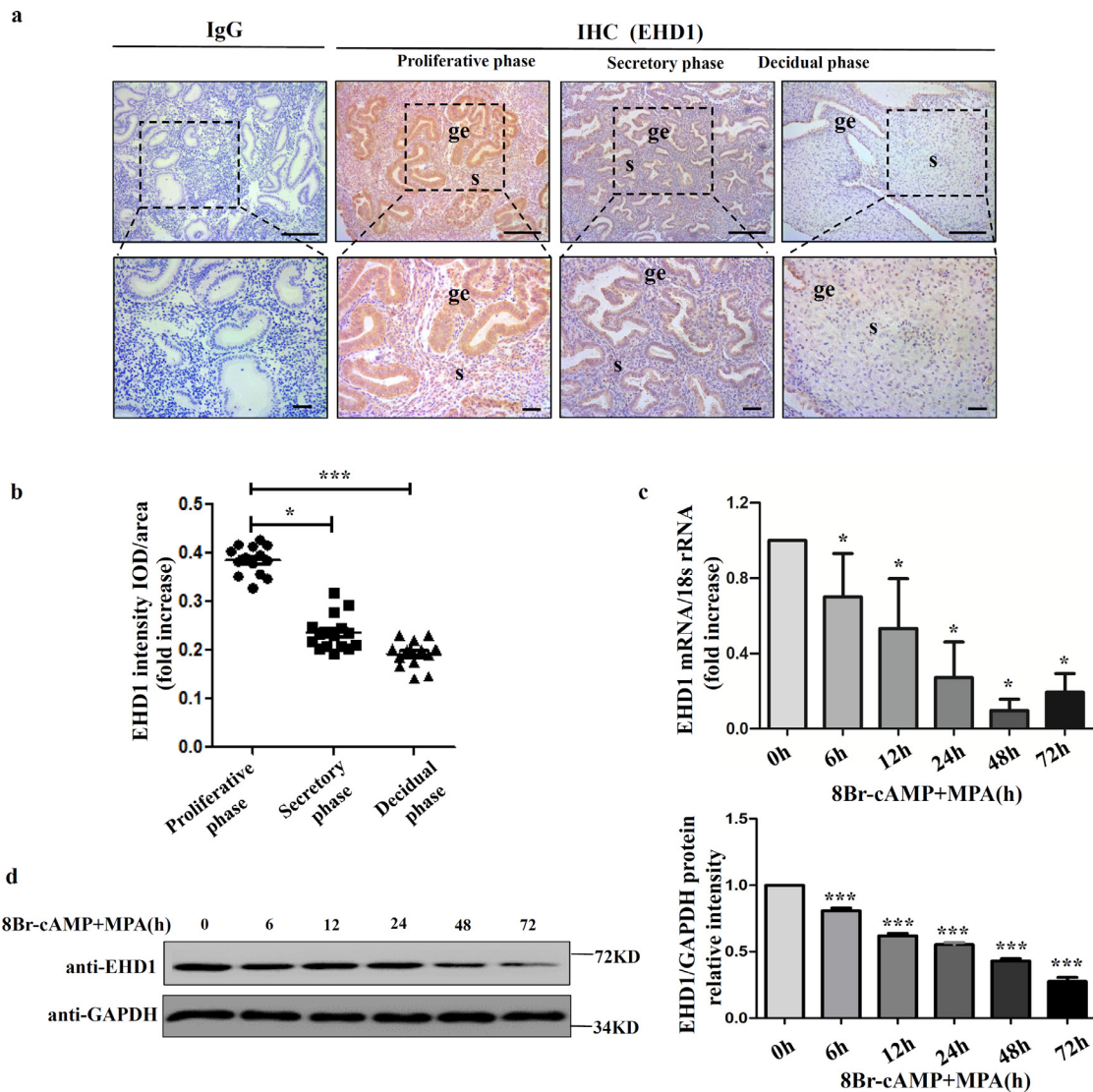
Fig. 3. EHD1 is mainly expressed in endometrial tissue in the uterus, and the levels decrease upon decidualization. (a, b) The protein expression levels of EHD1 in the proliferative, secretory and decidual phases were analyzed via real-time IHC staining. Ipwin32 software analysis was used to calculate the EHD1 integral optical density (IOD). Scale bars: 200 μm. s, stroma; ge, glandular epithelium. (c, d) The time-dependent effects of 8-Br-cAMP and MPA on EHD1 expression were determined by real-time PCR and Western blot analysis. $*p < 0.05$ compared to 0 h ($n = 3$, one-way analysis).

mRNA expression, and EHD1 protein expression was decreased by approximately 5-fold after 72 h of stimulation with 8-Br-cAMP and MPA (Fig. 3c and d).

3.5. EHD1 impairs HESC decidualization in vitro

The decrease in EHD1 expression during decidualization prompted us to examine the associated biological activities, and we found that HESCs with adenovirus-mediated overexpression of EHD1 (ad-Flag-EHD1) (Fig. 4b) showed significantly decreased expression levels of PRL and IGFBP1 mRNAs (Fig. 4b and c). Furthermore, EHD1 overexpression significantly inhibited decidual PRL production in a time-dependent manner (Fig. 4d). Decidualized HESCs displayed polygonal cell morphologies with a random distribution of F-actin filaments compared with nondecidualized HESCs. The overexpression of EHD1 induced a noticeable transformation from a round and randomly arranged shape to a long fibroblast-like phenotype (Fig. 4e). These findings indicated that increased expression of EHD1 might contribute to impaired decidualization in RIF patients.

3.6. RNA-Seq analysis of the HESCs in the normal, decidual and ad-Flag-EHD1 groups

To further identify the signaling pathway regulated by EHD1, we performed an RNA-Seq analysis of the HESCs in the normal, decidual and ad-Flag-EHD1 groups. In general, the three significant clusters (the blue, orange and red dots) from PCA analysis showed similar degrees of discrimination for different groups (Fig. 5a). More than 18,631 mRNA transcripts were detected in all the samples according to the defined expression threshold; genes above this threshold were included in the Venn graph calculation (Fig. 5b). Increased lines of evidence have demonstrated that the Wnt signaling pathway regulates endometrial stromal cell differentiation and thereby contributes to decidualization [22–24]. We selected 55 genes associated with the Wnt-associated pathway to visually observe their expression trends. The decidual group exhibited significant differences compared with the control group, and EHD1 overexpression reversed these changes, as demonstrated by the analysis of the hierarchical clustering algorithm (Fig. 5c).
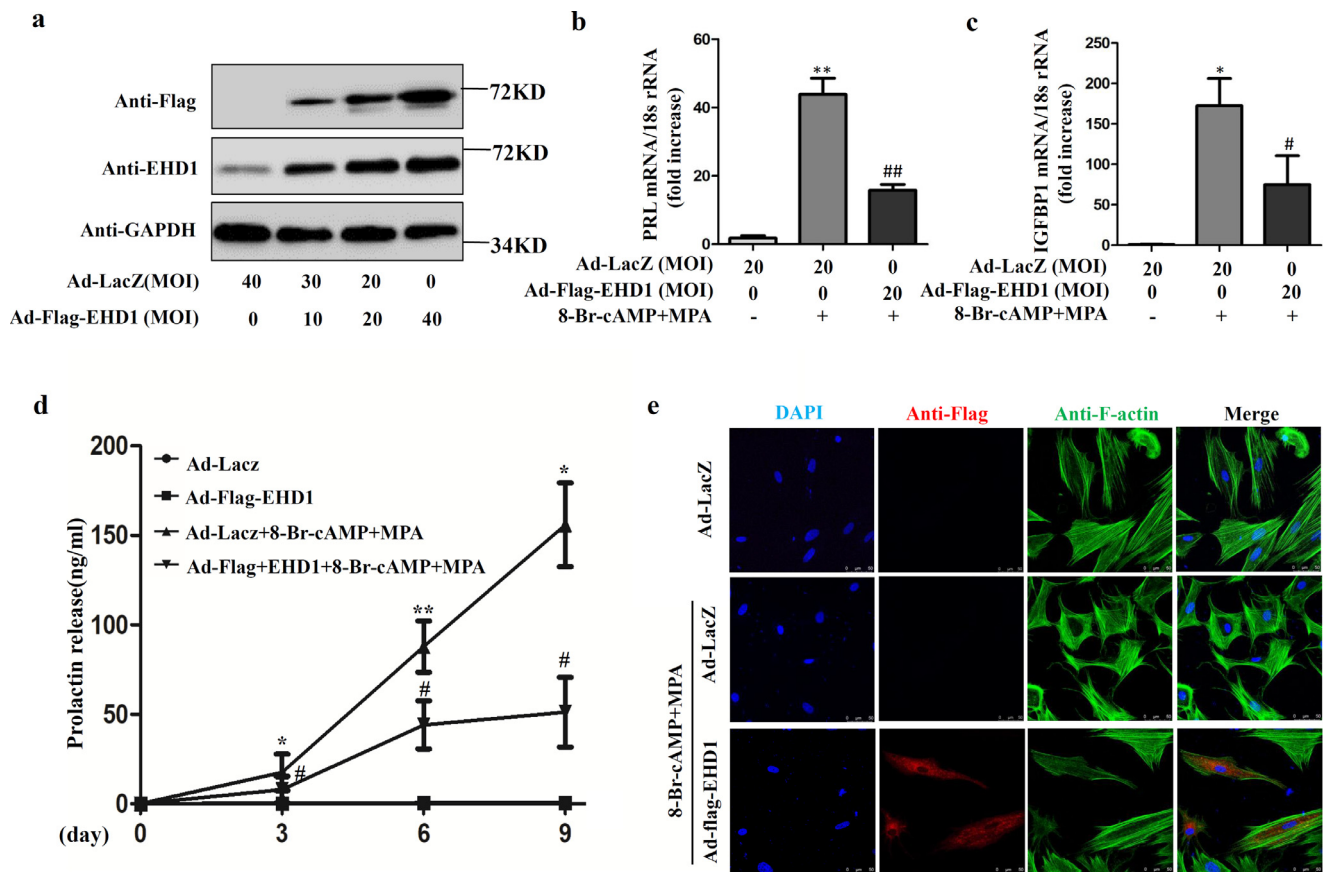
Fig. 4. **Overexpression of Ad-Flag-EHD1 inhibits HESC decidualization in vitro.** (a) HESCs were transduced with Ad-lacZ or Ad-Flag-EHD1 for two days at the MOI indicated, and EHD1 protein expression was measured using Western blot. (b, c) HESCs were infected with Ad-LacZ or Ad-Flag-EHD1 at the MOI indicated and treated with 8-Br-cAMP and MPA for three days, and PRL and IGFBP-1 mRNA levels were further measured using real-time PCR, $*p < 0.05$, $**p < 0.01$ compared with Ad-LacZ ($n = 3$, one-way analysis); $\#p < 0.05$, $\#\#p < 0.01$ compared with Ad-LacZ treated with 8-Br-cAMP and MPA ($n = 3$, one-way analysis). (d) HESCs were infected with the indicated adenoviruses for 24 h, followed by treatment with or without 8-Br-cAMP and MPA for an additional three, six, or nine days. Prolactin that was released into the medium was measured using ELISA, $*p < 0.05$, $**p < 0.01$ compared with Ad-LacZ alone ($n = 4$, one-way analysis); $\#p < 0.05$ compared with Ad-LacZ treated with 8-Br-cAMP and MPA ($n = 4$, one-way analysis). (e) Fluorescein isothiocyanate-labeled phalloidin was used to label actin filaments, and immunofluorescence was used to analyze the morphological transformation of HESCs.

The DEGs related to the Wnt pathway used in the GO analysis revealed their enrichment in multiple biological processes (Fig. 5d), and the KEGG pathway analysis also indicated that the genes were involved in pathways that regulate Wnt signaling (Fig. 5e). Our microarray data showed that the FPKM expression of Wnt4, CTNNB1, and LRP5 was upregulated and inhibited in the decidual and ad-Flag-EHD1 groups, respectively (Fig. 5f). Collectively, these results indicated that the EHD1-mediated Wnt4 signaling pathway is associated with cell decidualization.

3.7. EHD1 overexpression results in enhanced LRP5/6 endocytic recycling and mediates Wnt4 signaling pathway

A previous study provided evidence showing that Wnt signaling can be blocked by the rapid removal of LRP5/6 coreceptors via endocytosis [25]. Because EHD1 plays a critical role in receptor endocytosis and recycling, we investigated whether EHD1 regulates LRP5/6 proteins via a recycling route through the endocytic pathway and subsequently suppressed the Wnt4/β-catenin pathway during decidualization. Rabenosyn-11 (Rab11), which is a recycling endosome marker, was used to evaluate the endocytic function of LRP5/6 coreceptors [26] and our results revealed that the colocalization of LRP5/6 and Rab11 in HESCs was significantly enhanced by EHD1 overexpression (Fig. 6a and b).

We also found that EHD1 overexpression decreased Wnt4 and β-catenin protein levels (Fig. 7a). Importantly, the colocalization of

endogenous EHD1 and Wnt4 was enhanced in the endometrium of RIF patients (Fig. 7b), and the interaction between EHD1 and Wnt4 proteins in HEK293T cells and HESCs was further confirmed by coimmunoprecipitation (Co-IP) assays (Fig. 7c and d).

3.8. EHD1 impairs decidualization-dependent Wnt4 signaling

As shown by immunohistochemistry (Fig. 8a and b), Wnt4 signaling was decreased in the endometrium of the RIF group compared with that of the control group. Although EHD1 overexpression inhibited decidualization, treatment with a Wnt4 agonist (AG-L-67051) significantly increased the mRNA expression levels of PRL and IGFBP1 (Fig. 8c). Furthermore, the Wnt agonist treatment significantly promoted the secretion of decidual PRL from ad-Flag-EHD1-treated HESCs in a time-dependent manner (Fig. 8d).

4. Conclusion

Failure to implant might be a consequence of embryo or uterine factors [27]. Although the development of assisted reproductive technology has improved embryo quality, the embryo implantation rate remained relatively low over the past decade [28,29]. Increased lines of evidence demonstrate that impaired decidualization plays an important role in the pathophysiological process of RIF [2,29]. To explore the potential molecular mechanisms, biopsies at the mid-secretory phase were obtained to investigate the
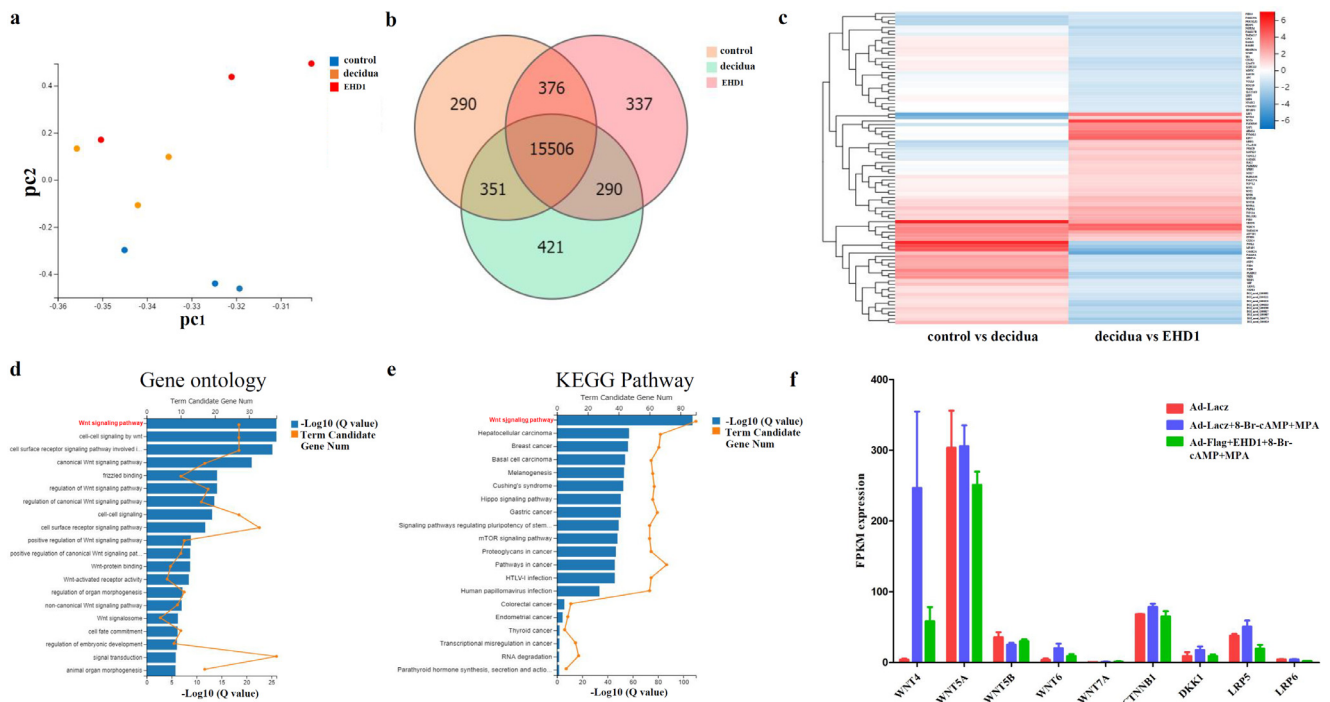
Fig. 5. EHD1 impairs HESC decidualization in vitro by repressing the Wnt4/β-catenin pathway. (a) 2D scatter plot of the principal component analysis (PCA) results. Samples in the control group (control), decidual (decidua) group and overexpression EHD1 group (EHD1) ($n = 3$) are represented by blue, orange and red dots, respectively. (b) Venn diagrams showing the overlapping genes and differentially expressed genes (DEGs) among the control, decidual, and EHD1 groups. (c) Hierarchical clustering of DEGs between different groups, the left panel with the control group vs the decidua group and the right panel with the decidua group vs the EHD1 group. (d, e) Gene Ontology (GO) functional classification of the DEGs and the KEGG pathway analysis between the decidual group and EHD1-overexpressing group. (f) FRKM expression by RNA-seq among the control, decidua, EHD1 groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
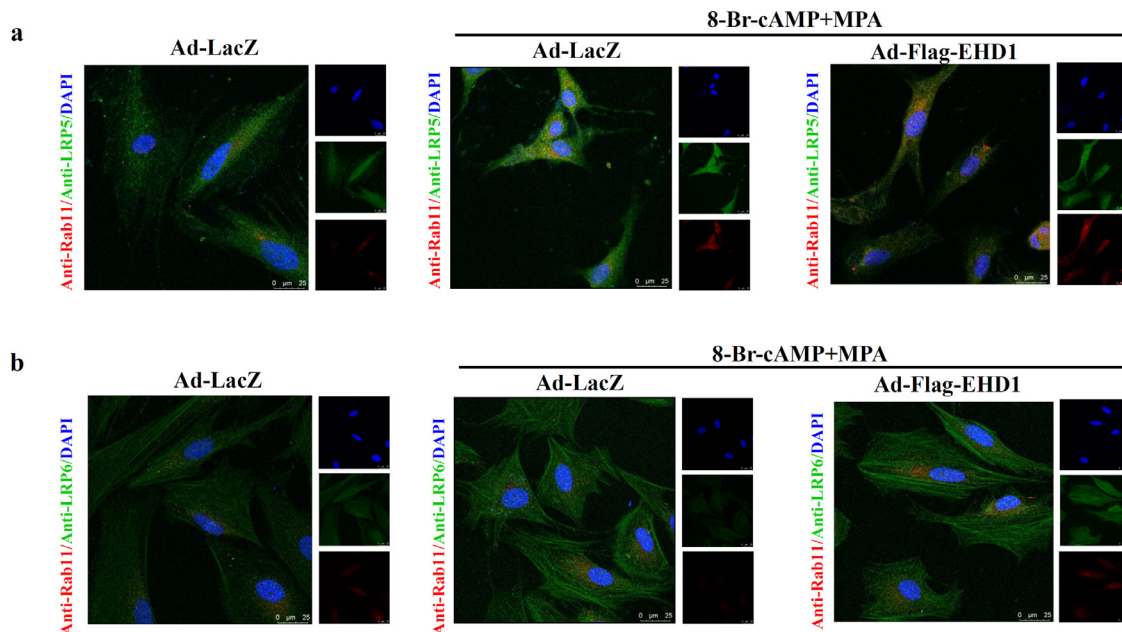

Fig. 6. Overexpression of EHD1 strengthens LRP5/6 endocytic recycling. (a) HESCs were infected with ad-LacZ or ad-Flag-EHD1 for 24 h, followed by treatment with or without 8-Br-cAMP and MPA for an additional three days. The colocalization of LRP5 and Rab11 were analyzed by immunofluorescence. (b) The colocalization of LRP6 and Rab11 in HESCs were also evaluated by immunofluorescence with Ad-LacZ or Ad-Flag-EHD1 indicated and treatment with 8-Br-cAMP and MPA.

differentially expressed genes at the transcriptional level between RIF patients and control subjects. According to the GO and KEGG analyses, a variety of membrane transport proteins associated with endocytic function showed abnormal changes. In particularly, the Hierarchical cluster analysis revealed that the EHD1 protein, which is encoded by a representative endocytic gene, exhibited the most

significant change. However, few studies have investigated the role of endocytic function in the endometrium and embryo implantation.

EHD proteins are highly conserved homologs of the *C. elegans* RME-1 protein, and mutation of the EH domain results in redistribution of the endocytic recycling compartment [30]. The mam-
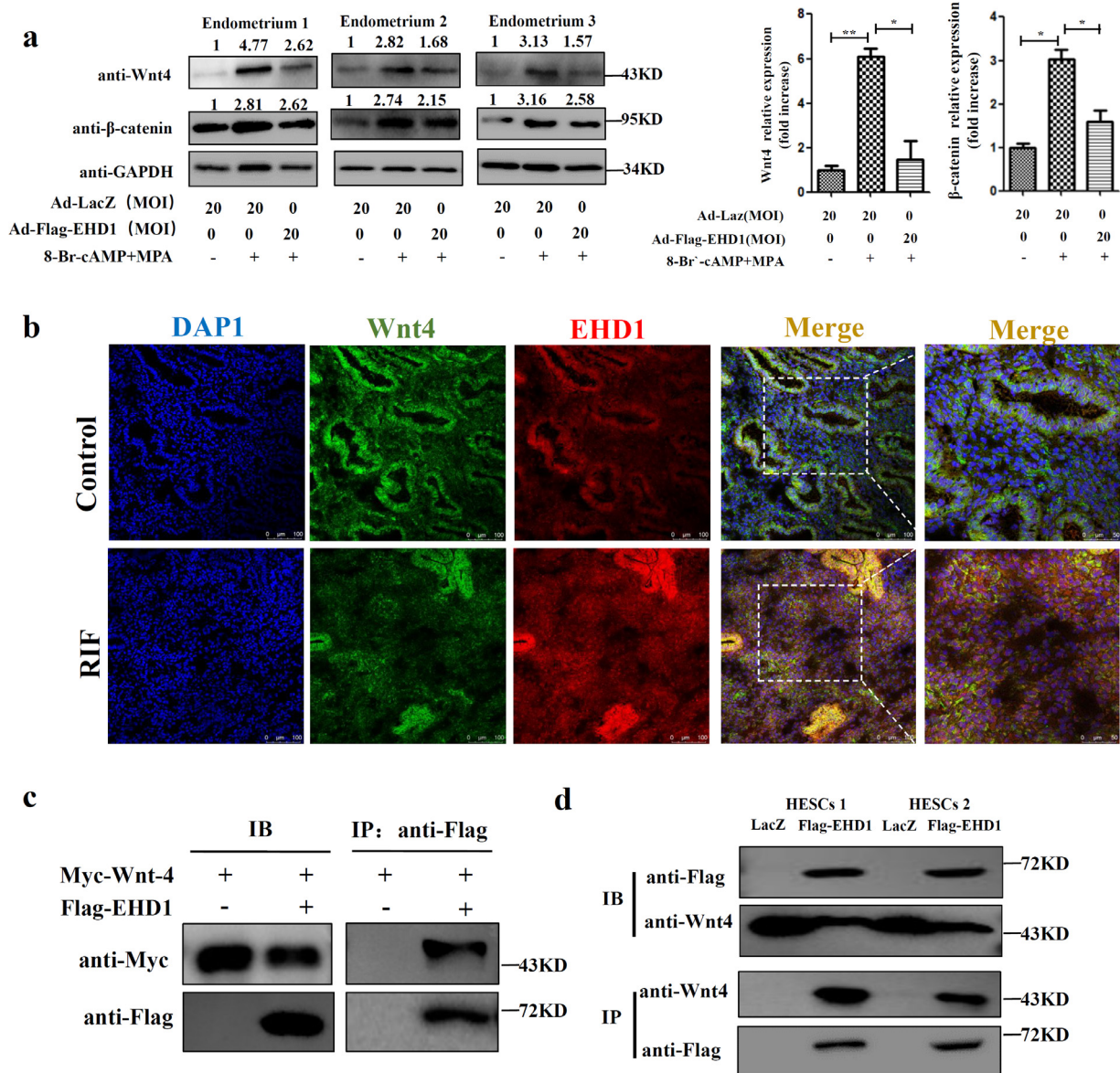
Fig. 7. The interaction between EHD1 and Wnt4. (a) The protein levels of Wnt4 and β-catenin with Ad-LacZ or Ad-Flag-EHD1 at the MOI indicated and after treatment with 8-Br-cAMP and MPA for three days were assessed by Western blot. $^*p < 0.05$, $^{**}p < 0.01$ compared with the control group or decidua group ($n = 3$, one-way analysis). (b) Images of the colocalization of EHD1 (red) and Wnt4 (green) in the control and RIF groups. Each of the two proteins was immunoprecipitated with anti-EHD1 antibody or anti-Wnt4 antibody from HEK293T cells (c) and HESCs (d), and the immunoprecipitates were subjected to Western blot analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

malian EHD protein regulates receptor recycling and interacts with Rab11 during the process of vesicle formation [26,31]. EHD1 is the most studied molecule among the EHD protein family, and previous studies have revealed its role in facilitating the endocytic recycling of insulin-like growth factor 1 receptor, insulin-responsive glucose transporter 4, and colony-stimulating factor-1 receptor and β2-adrenoceptor signaling [13,14,17,32]. The results obtained in our study indicate a novel function for EHD1 as a regulator of LRP 5/6 endocytosis, and this function involves the negative regulation of decidualization and contributes to embryo implantation failure.

Our study also showed that the expression of EHD1, rather than other EHD family proteins (EHD2/3/4), showed a periodical decrease during the menstrual cycle. We further showed that EHD1 is expressed in both endometrial epithelial cells and stroma cells in endometrial tissues, and the level of EHD1 expression in stroma cells was significantly increased in the RIF group compared with the control group. Decidualization of the human endometrium in-

volves a notable morphological and functional differentiation of human endometrial stromal cells, which is a crucial step in making a uterus receptive to an embryo [33]. cAMP-mediated protein kinase A (PKA) activation is critical for the initiation of decidualization and decidual PRL and IGFBP1 are major secretory products of the decidualized endometrium [34]. We showed that EHD1 expression gradually decreased in response to 8-Br-cAMP and MPA treatment in a time-dependent manner. The adenoviral-mediated overexpression of EHD1 markedly reduced decidual PRL and IGFBP1 expression, and impaired the cytoskeletal structure of decidualizing HESCs. These results suggest that the activation of EHD1 signaling is a crucial step contributing to the pathogenesis of RIF by inhibiting the process of decidualization.

Clinical research has suggested that the significant overexpression of Wnt4 signaling in the uterus during pregnancy plays important role in the regulation of endometrial stromal cell proliferation, survival and differentiation [23]. Female development in mammals
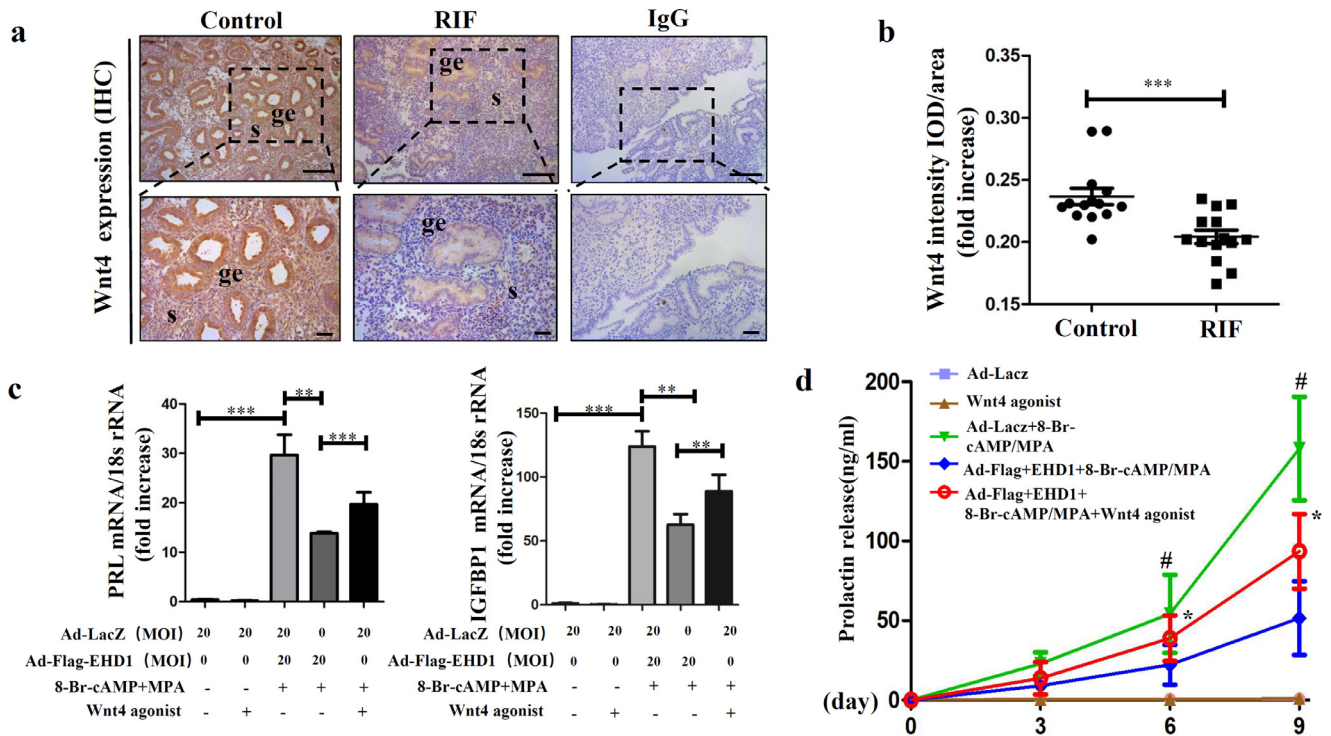
Fig. 8. Treatment with Wnt4 agonist improvs decidualization. (a, b) Immunohistochemical analysis was performed using Wnt4 antibody. Endometrial tissue samples from fertile women and RIF patients are shown at 100× (left panel) and 200× (right panel) magnification. ***$p < 0.001$ compared with the control group (student's t-test). Scale bars: 200 μm. s, stroma; ge, glandular epithelium. (c) HESCs were infected with Ad-Flag-EHD1 at the MOI indicated following Wnt4 agonist and 8-Br-cAMP/MPA treatment for three days, after which PRL and IGFBP-1 mRNA levels were measured using real-time PCR ($n = 3$, *$p < 0.01$, ***$p < 0.001$, one-way analysis). (d) HESCs were infected with the indicated adenoviruses for 24 h, followed by treatment with or without 8-Br-cAMP and MPA or Wnt4 agonist for an additional three, six, or nine days. Released prolactin was measured by ELISA, #$p < 0.05$, ##$p < 0.01$ compared with Ad-LacZ alone ($n = 3$, one-way analysis); * $p < 0.05$ compared with Ad-Flag-EHD1 treated with 8-Br-cAMP and MPA ($n = 3$, one-way analysis).

is regulated by Wnt4 signaling [35], and the inhibition of Wnt4/β-catenin signaling interferes with the process of embryo implantation [36]. Through animal studies, Franco et al. [24] demonstrated that the conditional knockout of Wnt4 in the uterus results in infertility and abnormal decidualization in mice. Herington et al. [37] used a mouse model of artificially stimulated decidualization to investigate the vital role of β-catenin during decidualization. Our study showed that the protein levels of Wnt4 and β-catenin declined by approximately 2-fold after treatment with ad-Flag-EHD1. A transcriptome analysis showed that the differentially expressed genes involved in the Wnt4/β-catenin signaling pathway exhibited notable changes in HESCs after the adenovirus-mediated overexpression of EHD1. In addition, we found that Wnt4 expression was significantly reduced in RIF patients, which confirmed that the EHD1-Wnt4/β-catenin pathway participates in the processes of decidualization and embryo implantation. Moreover, treatment with Wnt4 pathway agonists significantly increased the expression levels of PRL and IGFBP1. Thus, the identification of a positive agonist of Wnt4 will be beneficial to improving the treatments for RIF patients with impaired decidualization.

Although the overexpression of EHD1 inhibited decidualization, the precise mechanisms involving EHD1 and Wnt4 remain unclear. LRP5/6, which are single-spanning transmembrane proteins, are indispensable for the WNT/β-catenin pathway [38]. The binding of Wnt4 ligands to the cell-surface coreceptors LRP5/6 triggers the β-catenin-dependent pathway [39]. Because EHD1 plays a critical role in receptor endocytosis and recycling, we investigated whether EHD1 regulates LRP5/6 proteins via a recycling route through the endocytic pathway. Rab11, which is a recycling endosome marker, interacts with EHD1 through NPF-EH domain interactions and plays an important role in vesicle budding, de-

livery, tethering, and fusion [26]. The confocal microscopy analysis revealed that the colocalization of LRP5/6 with Rab11 in HESCs was markedly increased by EHD1 overexpression. Moreover, we found that EHD1 and Wnt4 bound to each other under physiological conditions and that this interaction was increased in RIF patients. Our data showed that EHD1-induced endocytosis can lead to a reduction in LRP5/6 and subsequently suppress the Wnt4/β-catenin pathway during decidualization in RIF.

RIF is a new gynecological disorder of unknown etiology (1). To investigate the potential mechanisms during RIF, various uterine pathologies including fibroids, endometrial polyps, congenital anomalies and intrauterine adhesions, should be excluded by ultrasonography and hysteroscopy, and women definitely diagnosed with endometriosis were excluded from the study. Although some patients show no clinical symptoms or a normal examination index, endometriosis might be present. To further investigate whether EHD1 participates in the pathogenesis of endometriosis, the expression of EHD1 in eutopic of endometriosis patients and the healthy endometrium of control subjects was evaluated by Western blotting. The results revealed no significant differences between the two groups (data not shown). Thus, the EHD1 signaling pathway might not be involved in the pathogenesis of endometriosis.

In conclusion, our study demonstrated that aberrant EHD1 signaling in the endometrium of RIF patients contributes to impaired decidualization and ultimately to implantation failure. EHD1 interacts with Wnt4, and this interaction leads to the suppression of Wnt4/β-catenin signaling and impaired decidualization (Supplementary Figure 3). Our study provides new insight into the molecular mechanism of EHD1 by showing that this protein might act as a regulator of the transport of cell membrane receptors

that are critical for decidualization. Therefore, the EHD1-Wnt4 signaling pathway has the potential to be an effective therapeutic target in the treatment of implantation failure caused by impaired decidualization.

Declaration of Competing Interest

We declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

CRediT authorship contribution statement

Quan Zhou: Data curation, Writing - review & editing. **Guijun Yan:** Data curation, Writing - review & editing. **Lijun Ding:** Data curation, Writing - review & editing. **Jingyu Liu:** Formal analysis, Writing - review & editing. **Xiaoying Yu:** Formal analysis, Writing - review & editing. **Shuangbo Kong:** Formal analysis, Writing - review & editing. **Mei Zhang:** Formal analysis, Writing - review & editing. **Yang Liu:** Formal analysis, Writing - review & editing. **Yue Jiang:** Formal analysis, Writing - review & editing. **Jianxin Sun:** Writing - review & editing. **Haixiang Sun:** Writing - review & editing.

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Data availability

The authors provide detailed description of methods and original data upon request. RNA-sequencing data that support the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) repository (http://www.ncbi.nlm.nih.gov/sra/) with accession number SRP224538 and SRP224679.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2019.10.018.

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